

GENETIC LOSS OF GAS6 INDUCES PLAQUE STABILITY IN EXPERIMENTAL ATHEROSCLEROSIS

Esther Lutgens^{1#}, Marc Tjwa^{2,3#}, Pablo Garcia de Frutos⁴, Erwin Wijnands¹, Linda Beckers¹, Björn Dahlbäck⁵, Mat Daemen¹, Peter Carmeliet^{2,3} and Lieve Moons^{2,3}.

1. Department of Pathology, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, Maastricht, The Netherlands.
2. Department for Transgene Technology and Gene Therapy, VIB, Leuven, Belgium.
3. Center for Transgene Technology and Gene Therapy (CTG), K.U.Leuven, Leuven, Belgium.
4. Instituto de Investigaciones Biomédicas de Barcelona, IIBB-IDIBAPS-CSIC, Barcelona, Spain.
5. Department of Clinical Chemistry, University of Lund, Malmo, Sweden.

#: these authors contributed equally to this study

Corresponding author: Peter Carmeliet (peter.carmeliet@med.kuleuven.be)

Center for Transgene Technology & Gene Therapy

Campus Gasthuisberg, Herestraat 49, box 912

B-3000, Leuven, Belgium

tel: +32-16-34.57.74; fax: +32-16-34.59.90

peter.carmeliet@med.kuleuven.be

ABSTRACT

The growth arrest-specific gene 6 (Gas6) plays a role in pro-atherogenic processes such as endothelial and leukocyte activation, smooth muscle cell migration and thrombosis, but its role in atherosclerosis remains uninvestigated. Here, we report that Gas6 is expressed in all stages of human and mouse atherosclerosis, in plaque endothelial cells, smooth muscle cells and macrophages. Gas6 expression is most abundant in lesions containing high amounts of macrophages, i.e. the thin fibrous cap atheroma and the ruptured plaque. Genetic loss of Gas6 does not affect the number and size of initial and advanced plaques in ApoE^{-/-} mice, but alters its plaque composition. Compared to Gas6^{+/+}:ApoE^{-/-} mice, initial and advanced plaques of Gas6^{-/-}:ApoE^{-/-} mice contained more smooth muscle cells, more collagen and developed smaller lipid cores, while the expression of TGF- β was increased. In addition, fewer macrophages were found in advanced plaques of Gas6^{-/-}:ApoE^{-/-} mice. Hence, loss of Gas6 promotes the formation of more stable atherosclerotic lesions by increasing plaque fibrosis and by attenuating plaque inflammation. These findings identify a role for Gas6 in plaque composition and stability.

INTRODUCTION

The growth arrest-specific gene 6 (Gas6) was discovered more than a decade ago , but its role in the vessel wall still remains poorly characterized. Gas6 binds three distinct receptor tyrosine kinases, i.e. Axl, Tyro3 and Mer (reviewed in [2]). Gas6 is composed of a N-terminal gamma-carboxy-glutamic acid domain (Gla-domain), a loop region, four EGF-like repeats, and a C-terminal SHBG-like domain (steroid hormone binding globulin-like domain), and was discovered as a homologue of the anti-coagulant Protein S . Originally identified in fibroblasts, Gas6 appears to be expressed in various cell types, including hematopoietic cells [5]. In erythroid progenitors and erythroblasts, Gas6 is a strong survival factor and enhances the effects of erythropoietin [6]. In platelets, Gas6 and its receptors amplify murine platelet activation and aggregation in a feedback loop [7-10]. Leukocytes also express Gas6 and its receptors, especially in conditions of inflammation and repair [11-14], and several reports document that Gas6 exerts both positive and negative effects on leukocyte activation . Recently, we found that Gas6 is able to amplify endothelial function and that it promotes the sequestration of platelets and leukocytes to the activated endothelium and the formation and sequestration of platelet-leukocyte aggregates [18]. Moreover, in animal models of inflammation, Gas6 enhances leukocyte extravasation, inflammation and thrombosis [18].

Several lines of evidence suggest that Gas6 may play a role in the vessel wall. Indeed, Gas6 and its receptors are expressed in smooth muscle cells (SMCs), fibroblasts, pericytes and endothelial cells (ECs), and the expression of Gas6 and its receptors is upregulated and activation of Gas6 receptors is increased in conditions of vascular injury, inflammation and repair [1, 11, 14, 19-23]. Gas6 alone induces modest mitogenic effects in SMCs and fibroblasts [22, 24-27], but potentiates the effect of other mitogens [22, 28, 29]. Gas6 also stimulates the migration [30] and survival of these cells [21, 25, 27, 31], and affects their differentiation [32]. Notably, the Gas6-mediated survival pathway in SMCs is a known mechanism of how HMGcoA-reductase inhibitors are able to prevent vascular calcification [33, 34]. Moreover, deficiency of Axl decreases intimal thickening in cuff injury and partial ligation models [14, 23], suggesting a role for Gas6 in neointima formation and atherosclerosis.

However, little is known about the role of Gas6 in atherosclerosis. Indeed, one *in vitro* study shows that Gas6 induces the expression of the scavenger receptor class A in

SMCs, and that the expression of Gas6 is upregulated by oxidized LDL [20], but a role of Gas6 in atherosclerosis *in vivo* has not been documented yet. We therefore studied, by crossbreeding our previously generated Gas6^{-/-} mice with ApoE^{-/-} mice (Gas6^{-/-}:ApoE^{-/-} mice), whether Gas6 might play a role in atherosclerosis *in vivo*.

METHODS

Human atherosclerosis

Atherosclerotic plaques of all stages (pathological intimal thickening, intimal xanthoma, fibrous cap atheroma, thin fibrous cap atheroma, ruptured plaque) were obtained from the carotid artery bifurcations during autopsy. Patients were 63-75 years old, and this group consisted of 6 males and 2 females. Material was collected in compliance with institutional ethical guidelines in accordance with the Helsinki Declaration of 1975, as revised in 1983. After excision of the carotid artery bifurcations, arteries were fixed overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin as described .

Experimentally induced atherosclerosis in mice

The generation of $\text{Gas6}^{-/-}$ mice has been described [7]. To generate $\text{Gas6}^{-/-}:\text{apoE}^{-/-}$ mice, $\text{Gas6}^{-/-}$ (50%129/Sv x 50%Swiss) mice were intercrossed with $\text{apoE}^{-/-}$ mice (100% C57/Bl6). Heterozygote matings sired $\text{Gas6}^{+/+}:\text{apoE}^{-/-}$ and $\text{Gas6}^{-/-}:\text{apoE}^{-/-}$ mice (50% C57/Bl6 / 25% 129/Sv / 25%Swiss) at the expected Mendelian inheritance frequency (6.25%). No increased morbidity, signs of developmental problems or mortality were observed in $\text{Gas6}^{-/-}:\text{ApoE}^{-/-}$ mice. Housing and all experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the K.U. Leuven (Leuven, Belgium).

$\text{Gas6}^{-/-}:\text{ApoE}^{-/-}$ ($N=12$) and $\text{Gas6}^{+/+}:\text{ApoE}^{-/-}$ mice ($N=12$) were fed a normal chow diet for 23 weeks. Two weeks before the end of the experimental period, 5'-bromo-2'-deoxyuridine (BrdU, Serva Heidelberg, Germany, infusion rate 13 mg/kg/day) was infused using osmotic minipumps (Alzet, 2001). After the experimental period, mice were sacrificed, blood was withdrawn and the arterial tree was excised. Plasma cholesterol levels were determined in duplicate by using colorimetric assays (CHOD-PAP 1442341; Boehringer Mannheim). For histological analysis, the aortic arch, including its main branch points (brachiocephalic artery, left carotid artery, left subclavian artery) was processed as described [36]. Blood pressure measurements were performed using high-fidelity micromanometers as described previously.[37]

Histological and immunohistochemical analysis

For gross histological and morphometric analysis, 4 μ m paraffin sections were stained with haematoxylin and eosin (H&E). Atherosclerotic lesions were divided into initial lesions (pathological intimal thickenings and intimal xanthomas) and advanced lesions ((thin) fibrous cap atheromas) according to the classification of Virmani *et al.* [38]. In the mouse aortic arches, total plaque area was determined using the mean of total plaque area of 4 aortic arches. Individual initial and individual advanced plaque area were determined by dividing the sum of all individual initial or advanced areas by the total number of initial or advanced plaques.

For the visualization of Gas6 in human and mouse atherosclerosis, paraffin sections were immunolabeled with antibodies to Gas6 (rabbit polyclonal, 1:50, Santa Cruz). To assess the phenotype of Gas6-positive cells in the plaque, sections were double-labeled with cell-type specific antibodies for macrophages (mouse tissue: anti-Mac3, rat polyclonal, 1:30, Pharmingen; human tissue: CD68, mouse monoclonal, 1:500, DAKO), smooth muscle cells (anti-smooth muscle alpha-actin (ASMA), mouse monoclonal, 1:3000, Sigma) or endothelial cells (von Willebrand factor (vWF), rabbit polyclonal, 1:500, Dako) as described [35]. To determine plaque composition, sections of mouse atherosclerotic plaques were immunolabeled via standard protocols, using the appropriate biotin labeled secondary antibodies, and the avidin biotin complex method (DAKO, ABC^{ap} or ABC^{HRP} kit). The vectastain alkaline phosphatase kit (vectastain red/blue), or DAB (sigma) were used as chromogens. For counterstaining of the nuclei, hematoxylin was used. Mouse sections were labeled with anti-SMA, anti-Mac3, anti-vWF and anti-CD3 (rabbit polyclonal, 1:200, Dako) to identify T-lymphocytes. An anti-TGF- β antibody (LAP-TGF- β 1, rabbit polyclonal, 1:100 dilution, R&D Systems) was used to detect both latent and active TGF- β . DNA-synthesizing cells were identified using anti-BrdU (Mas 250b, 1:30, Harlan sera labs), as described previously [36]. A TUNEL assay (Boehringer Mannheim) was used to determine apoptosis. Negative controls for all immunostainings were performed by omitting the primary antibody. Collagen deposition was determined using a Sirius Red staining. Morphometric analysis was performed on a Zeiss Axioplan 2 imaging microscope equipped with a Axiocam HrC camera and KS300 morphometry software (Zeiss), as described [35, 39].

Statistical analysis

The data are represented as mean \pm SEM. Gas6^{-/-}:ApoE^{-/-} mice were compared with Gas6^{+/+}:ApoE^{-/-} mice using a non-parametric Mann-Whitney U test. Data were considered statistically significant at $P < 0.05$.

RESULTS

EXPRESSION OF GAS6 IN HUMAN AND MURINE ATHEROSCLEROTIC PLAQUES

In human atherosclerotic lesions, Gas6 was found to be expressed in macrophages, smooth muscle cells (SMCs) and endothelial cells in all stages of atherosclerosis. In pathological intimal thickenings and intimal xanthomas, Gas6 was most abundantly expressed in the foam cell rich macrophage area in the intima. In fibrous cap atheromas, thin fibrous cap atheromas and ruptured plaques, Gas6 was expressed on surface endothelial cells, macrophages in the shoulder region and in SMCs in the fibrous cap region. The expression of Gas6 was highest in lesions containing high amounts of macrophages, i.e. the thin fibrous cap atheromas and ruptured plaques (Figure 1 a-e).

A similar expression pattern was observed in mouse atherosclerotic plaques. Gas6 was present in the normal arterial wall and expressed in plaque macrophages, SMCs and endothelial cells of all lesion types (Figure 2 a-d). Gas6 was predominantly present in macrophage rich plaques. No Gas6 was detected in plaques of Gas6^{-/-}:ApoE^{-/-} mice (not shown). Thus, Gas6 is expressed in plaque ECs, SMCs and macrophages.

LOSS OF GAS6 DOES NOT AFFECT SYSTEMIC PARAMETERS

Gas6^{+/+}:ApoE^{-/-} and Gas6^{-/-}:ApoE^{-/-} mice were fed normal chow and analyzed for atherosclerosis development at 23 weeks of age. No genotypic differences were found in plasma cholesterol levels (mmol/L: 7.0 ± 0.3 in Gas6^{+/+}:ApoE^{-/-} mice *versus* 7.5 ± 0.4 in Gas6^{-/-}:ApoE^{-/-} mice; N=12; P=NS). In addition, peripheral blood profiles (including leukocyte and platelet counts), body weights and heart weights were similar in both genotypes (not shown). Blood pressure or heart rate did not differ between both genotypes (data not shown). By macro- and microscopic analysis, no genotypic differences were found in any other organs, including liver, spleen, lung and brain (not shown).

LOSS OF GAS6 DOES NOT AFFECT SIZE AND NUMBER OF INITIAL AND ADVANCED PLAQUES

We then analyzed, at 23 weeks of age, the number and size of individual plaques in the aortic arch of Gas6^{+/+}:ApoE^{-/-} and Gas6^{-/-}:ApoE^{-/-} mice, fed a normal chow. Plaques were categorized as initial or advanced plaques and, in total, 49 lesions in both genotypes (N=12) were analyzed. This analysis revealed that the sizes of initial and advanced plaques, as well as total plaque area were not different between Gas6^{+/+}:ApoE^{-/-} and Gas6^{-/-}:

^{-/-}:ApoE^{-/-} mice (Table 1; Figure 3a,b). In addition, we found no genotypic differences in the number of plaques (Table 1; Figure 3a,b).

LOSS OF GAS6 ALTERS THE COMPOSITION OF INITIAL PLAQUES

We next studied, in Gas6^{+/+}:ApoE^{-/-} and Gas6^{-/-}:ApoE^{-/-} mice, the composition of the initial plaques (pathological intima thickenings and intimal xanthomas). As expected, in Gas6^{+/+}:ApoE^{-/-} mice, initial lesions mostly consisted of infiltrated macrophages, some T-lymphocytes and SMCs, and a limited amount of collagen deposition (Table 1; Figure 4a). In contrast, in Gas6^{-/-}:ApoE^{-/-} mice, early lesions contained more collagen (Table 1; Figure 4b). Consistent herewith, more SMCs had infiltrated the initial lesions of Gas6^{-/-}:ApoE^{-/-} mice, while the number of macrophages and T-lymphocytes were similar (Table 1). Hence, loss of Gas6 promotes the formation of more fibrotic pathological intima thickenings and intimal xanthomas.

LOSS OF GAS6 ALTERS THE COMPOSITION OF ADVANCED PLAQUES

We also investigated the composition of complex advanced plaques ((thin) fibrous cap atheromas) in Gas6^{+/+}:ApoE^{-/-} and Gas6^{-/-}:ApoE^{-/-} mice. As expected, in Gas6^{+/+}:ApoE^{-/-} mice, advanced plaques contained a large number of macrophages, some T-lymphocytes, a few large central necrotic lipid cores and an overlying fibrous cap (Table 1; Figure 4c). In contrast, in Gas6^{-/-}:ApoE^{-/-} mice, the composition of advanced plaques was markedly different.

Compared to Gas6^{+/+}:ApoE^{-/-} mice, SMC-content had increased in advanced plaques of Gas6^{-/-}:ApoE^{-/-} mice (Table 1; Figure 4c,d). SMCs in Gas6^{+/+}:ApoE^{-/-} mice were confined to the shoulder and fibrous cap region, while SMCs in the lesions of Gas6^{-/-}:ApoE^{-/-} mice were not only present in the shoulder and fibrous cap, but lay also scattered throughout the lesion and surrounded the lipid cores (Figure 4c,d). Consistent herewith, advanced lesions of Gas6^{-/-}:ApoE^{-/-} mice contained more collagen throughout the plaques (Table 1; Figure 3e,f), suggesting that SMCs were responsible for the increased production and deposition of collagen in plaques of Gas6^{-/-}:ApoE^{-/-} mice. Taken together, loss of Gas6 promotes the formation of more fibrotic atheromata.

Advanced lesions of Gas6^{-/-}:ApoE^{-/-} mice did not contain the usual large central lipid cores but, instead, contained numerous smaller lipid cores, likely as a result of the

increased deposition of collagen, scattered throughout the plaque (Table 1; Figure 3g,h). Moreover, in advanced plaques of Gas6^{-/-}:ApoE^{-/-} mice, the lipid core content was reduced (Table 1; Figure 4g,h). Gas6 deficiency did not affect plaque calcification, as analyzed after von Kossa staining. Calcified areas were observed in 4/22 advanced plaques in the Gas6^{-/-}:ApoE^{-/-}, and 4/25 plaques of the Gas6^{+/+}:ApoE^{-/-} mice.

Moreover, fewer macrophages were found in advanced lesions of Gas6^{-/-}:ApoE^{-/-} mice (Table 1). Though, admittedly, the macrophage counts in plaques of Gas6^{-/-}:ApoE^{-/-} mice were only modestly reduced, this phenotype appeared to be specific, since no genotypic differences were found in the number of infiltrated T-lymphocytes (Table 1). Overall, these data suggest that loss of Gas6 promotes the formation of more stable atherosclerotic lesions.

LOSS OF GAS6 INCREASES THE EXPRESSION OF THE PRO-FIBROTIC CYTOKINE TGF- β

Prompted by the observation that plaques of Gas6^{-/-}:ApoE^{-/-} mice contained more SMCs and collagen, we further investigated whether the pro-fibrotic cytokine TGF- β was involved, which is known to promote collagen deposition in atherosclerotic plaques [39-42]. Immuno-staining with an anti-TGF- β antibody, recognizing both latent and active forms, revealed that, compared to Gas6^{+/+}:ApoE^{-/-} mice, the expression of TGF- β was increased in atherosclerotic lesions of Gas6^{-/-}:ApoE^{-/-} mice (Figure 4i,j). As expected, TGF- β immunoreactivity was largely confined to SMCs and macrophages (data not shown). Hence, loss of Gas6 promotes the formation of more fibrotic plaques, at least in part, by increased TGF- β expression.

LOSS OF GAS6 DOES NOT AFFECT PROLIFERATION AND APOPTOSIS IN PLAQUES

Since Gas6 plays a role in cell survival, growth and apoptosis [21, 22, 24-29, 31], we counted the number of proliferating and apoptotic cells in initial and advanced lesions of Gas6^{+/+}:ApoE^{-/-} and Gas6^{-/-}:ApoE^{-/-} mice. BrdU- and TUNEL-positive cells were largely identified as SMCs and inflammatory cells (not shown). No genotypic differences were found in the extent of cellular proliferation (% BrdU⁺ cells over total cells: initial lesions: $5.9 \pm 2.1\%$ in Gas6^{-/-}:ApoE^{-/-} mice *versus* $6.3 \pm 3.3\%$ in Gas6^{+/+}:ApoE^{-/-} mice; advanced lesions: $3.1 \pm 1.7\%$ in Gas6^{-/-}:ApoE^{-/-} mice *versus* $2.9 \pm 1.4\%$ in Gas6^{+/+}:ApoE^{-/-} mice; $N=49$; $P=NS$), or apoptosis (%TUNEL⁺ cells over total cells: initial lesions: $0.4 \pm 0.2\%$ in

Gas6^{-/-}:ApoE^{-/-} mice *versus* 0.4 ± 0.3% in Gas6^{+/+}:ApoE^{-/-} mice; advanced lesions: 1.3 ± 0.7% in Gas6^{-/-}:ApoE^{-/-} mice *versus* 1.2 ± 0.2% in Gas6^{+/+}:ApoE^{-/-} mice; N=49; P=NS). Thus, loss of Gas6 did not affect cell turnover parameters of SMCs and inflammatory cells in initial and advanced plaques.

DISCUSSION

The role of Gas6 in atherosclerosis has not been characterized in detail before. Here, we report that Gas6 is expressed in all stages of human and murine atherosclerosis, and most abundantly in the macrophage-rich areas of the plaque. Consequently, the highest expression of Gas6 was found in thin fibrous cap atheromas. These data suggest an involvement of Gas6 in atherosclerosis and plaque vulnerability.

Indeed, in the absence of Gas6, we found that, in a mouse model of atherosclerosis, loss of Gas6 promotes the formation of more fibrotic atherosclerotic lesions and attenuates persistent inflammation, though the number and size of initial and advanced plaques and the systemic cholesterol levels were unaffected. These findings provide novel insights in the role of Gas6 in atherosclerosis, and indicate a role for Gas6 in plaque progression.

The most remarkable finding was the presence of more fibrotic atherosclerotic lesions in Gas6^{-/-}:ApoE^{-/-} mice, suggesting the formation of more stable plaques according to Virmani et al. Indeed, in initial and advanced plaques, loss of Gas6 induced the expression of TGF- β in SMCs and inflammatory cells of the plaque. The increase in SMC-content, together with the increase of the pro-fibrotic cytokine TGF- β , explains the increased deposition of collagen in absence of Gas6.

Interestingly, TGF- β not only acts on SMCs, but is also a potent anti-inflammatory cytokine secreted by (subsets of) T-cells and macrophages. Indeed, absence of TGF- β signaling in CD4⁺ cells (CD4⁺TGF- β RIIDN) results in a profound increase in atherosclerosis and in the development of an inflammatory rich, collagen-poor, unstable atherosclerotic plaque phenotype in ApoE^{-/-} mice [41, 42]. Therefore, the increased expression of TGF- β in inflammatory cells in the plaque, as observed in atherosclerotic lesions of our Gas6^{-/-}:ApoE^{-/-} mice, may contribute to a reduced influx and reduced activation of inflammatory cells.

Previous reports showed that, *in vitro*, Gas6 might exert positive effects of Gas6 on SMC proliferation, migration and survival [22, 28, 29]. Therefore, our *in vivo* findings that loss of Gas6 increases the amount of SMCs and collagen in the plaque are somehow surprising. In the absence of Gas6, proliferation and apoptosis indices of plaque SMCs were unaltered, suggesting that loss of Gas6 might have promoted the infiltration of SMCs into the plaques. Since inflammatory cytokines and proteases, secreted by plaque

macrophages and T-lymphocytes, favor extra-cellular matrix degradation [43], and since fewer macrophages were present in advanced Gas6^{-/-}:ApoE^{-/-} plaques, we speculate that reduced levels of inflammatory mediators might have promoted SMC infiltration and, secondarily, deposition of matrix components, and thus a more stable plaque phenotype. However, it is likely that Gas6 has additional effects on SMCs since, in early lesions of Gas6^{-/-}:ApoE^{-/-} mice, the number of SMCs was increased, yet infiltration of macrophages was normal. Future studies will address these issues.

Another interesting finding is the observation that, in advanced plaques of Gas6^{-/-}:ApoE^{-/-} mice, fewer lipid cores were formed and failed to merge into large central lipid cores. Indeed, according to AHA guidelines [38, 44], the presence of small lipid cores, as in plaques of Gas6^{-/-}:ApoE^{-/-} mice, is indicative of more stable plaques. Since Gas6 is known to upregulate the expression of scavenger receptor A in SMCs and oxidized LDL upregulates Gas6 expression [20], it might play a role in oxidized LDL uptake and foam cell formation. In addition, the increased collagen deposition and fibrotic plaque structure likely explains the impaired merging of small lipid cores into large central lipid cores in plaques of Gas6^{-/-}:ApoE^{-/-} mice.

In conclusion, our data show that loss of Gas6 promotes plaque stability by increasing plaque fibrosis and by attenuating plaque inflammation.

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TABLE 1: Atherogenesis in Gas6^{+/+}:ApoE^{-/-} and Gas6^{-/-}:ApoE^{-/-} mice.

	Gas6 ^{+/+} :ApoE ^{-/-} mice	Gas6 ^{-/-} :ApoE ^{-/-} mice
PLAQUE SIZES (x 10³ μm²)		
Individual initial plaque area	9.7 ± 1.6	11.9 ± 1.6
Individual advanced plaque area	65.3 ± 11.1	61.8 ± 11.6
Total plaque area (= mean plaque area/aortic arch/mouse)	122.2 ± 51.0	174.1 ± 119.4
NUMBER OF PLAQUES PER AORTIC ARCH	2.8 ± 0.8	2.9 ± 0.6
MAC3⁺ MACROPHAGES (CELLS PER PLAQUE)		
Initial plaques	28 ± 6	31 ± 4
Advanced plaques	185 ± 9	133 ± 11*
MAC3⁺ MACROPHAGES (% OF TOTAL CELLS)		
Initial plaques	68 ± 3	73 ± 3
Advanced plaques	54 ± 3	43 ± 5*
CD3⁺ LYMPHOCYTES (CELLS PER PLAQUE)		
Initial plaques	0.3 ± 0.2	0.2 ± 0.1
Advanced plaques	2.5 ± 0.5	2.0 ± 0.4
CD3⁺ LYMPHOCYTES (% OF TOTAL CELLS)		
Initial plaques	0.7 ± 0.4	1.0 ± 0.5
Advanced plaques	0.9 ± 0.3	1.1 ± 0.4
SMA⁺ SMCs (AREA PER PLAQUE IN μm²)		
Initial plaques	9 ± 6	22 ± 6*
Advanced plaques	539 ± 180	1387 ± 223*
SMA⁺ SMCs (% OF PLAQUE AREA)		
Initial plaques	0.3 ± 0.2	1.1 ± 0.9*
Advanced plaques	1.4 ± 0.5	4.9 ± 2.2*
COLLAGEN DEPOSITION (% OF PLAQUE AREA)		
Initial plaques	0.1 ± 0.1	7 ± 3*
Advanced plaques	22 ± 5	49 ± 6*
LIPID CORE IN ADVANCED PLAQUES		
Content (% of plaque area)	34 ± 4	23 ± 10*
Number of lipid cores per plaque	1.2 ± 0.1	2.1 ± 0.3*

The data represent the mean ± SEM of the average total plaque area per aortic arch, the size of individual plaques, and the average number of plaques per aortic arch (*N*=12 for Gas6^{+/+}:ApoE^{-/-} mice and *N*=12 for Gas6^{-/-}:ApoE^{-/-} mice). *: *P*<0.05 versus Gas6^{+/+}:ApoE^{-/-} mice.

FIGURE LEGENDS

FIGURE 1: EXPRESSION OF GAS6 IN HUMAN ATHEROSCLEROTIC VESSELS.

a, Gas6 (red) is present in the intima of a pathological intimal thickening (PIT), predominantly in the macrophage-rich area. **b**, Gas6 (red) is predominantly present in macrophage-rich areas of the shoulder regions and fibrous cap region of a fibrous cap atheroma. LC:..... **c-e**, Double immunostainings revealed that Gas6 (blue) is detectable in ASMA⁺ smooth muscle cells (arrowheads show 2 typical examples; **c**), in Mac3⁺ macrophages (arrowhead; **d**) and in vWF⁺ endothelial cells (arrowhead; **e**). Magnification bars: 50 μ m in panels **a,b**; 10 μ m in panels **c-e**. L: lumen, M: media.

FIGURE 2: EXPRESSION OF GAS6 IN MURINE ATHEROSCLEROTIC VESSELS.

a, Gas6 (red) is present in atherosclerotic lesions in the aortic arch of Gas6^{+/+}:ApoE^{-/-} mice. **b-d**, Double immunostainings revealed that Gas6 (blue) is detectable in ASMA⁺ smooth muscle cells (arrowheads show 2 typical examples; **b**) in Mac3⁺ macrophages (arrowheads show 2 typical examples; **c**), and in vWF⁺ endothelial cells (**d**). L indicates vessel lumen. Magnification bars: 50 μ m in panel **a**; 10 μ m in panel **b-d**.

FIGURE 3: NORMAL PLAQUE NUMBER AND SIZE IN GAS6^{-/-}:APOE^{-/-} MICE.

a,b, Longitudinal sections throughout the aortic arch including the brachiocephalic artery and right carotid artery (brachiocephalic trunk), the left carotid artery and left subclavian artery of Gas6^{+/+}:ApoE^{-/-} (**a**) and Gas6^{-/-}:ApoE^{-/-} (**b**) mice showing the absence of genotypic differences in plaque number and size. AA: aorta ascendens; BCT: brachiocephalic trunk; LCCA: left common carotid artery; LSA: left subclavian artery.

FIGURE 4: ALTERED COMPOSITION OF INITIAL AND ADVANCED PLAQUES IN GAS6^{-/-}:APOE^{-/-} MICE.

a,b, Sirius Red staining (SR; red) showing collagen deposition in initial plaques. Compared to Gas6^{+/+}:ApoE^{-/-} mice (**a**), more collagen was found in initial lesions of Gas6^{-/-}:ApoE^{-/-} mice (**b**). **c,d**, ASMA staining (brown) revealed that, compared to Gas6^{+/+}:ApoE^{-/-} mice (**c**), more SMCs had infiltrated the advanced lesions of Gas6^{-/-}:ApoE^{-/-} mice (**d**). Note that, compared to Gas6^{+/+}:ApoE^{-/-} mice (**c**), ASMA-positive cells were distributed throughout the lesions of Gas6^{-/-}:ApoE^{-/-} mice (**d**). **e,f**, Sirius Red staining (SR; red) showing collagen

deposition in advanced plaques. Compared to Gas6^{+/+}:ApoE^{-/-} mice (e), more collagen was found in advanced lesions of Gas6^{-/-}:ApoE^{-/-} mice (f). **g,h**, H&E staining revealed the presence of large central lipid cores (black arrow) in advanced lesions of Gas6^{+/+}:ApoE^{-/-} mice (g). By contrast, in Gas6^{-/-}:ApoE^{-/-} mice, small lipid cores (black arrows) were found, which seemed to be encapsulated by collagen deposits (h). **i,j**, TGF-β staining (red) revealed, compared to Gas6^{+/+}:ApoE^{-/-} mice (i), more immunoreactivity in advanced lesions of Gas6^{-/-}:ApoE^{-/-} mice (j). Note that, in Gas6^{-/-}:ApoE^{-/-} mice, many TGF-β-expressing cells were found in the central region (j). Inset: detailed view showing a macrophage expressing TGF-β (arrowhead; j). L indicates vessel lumen. Magnification bars: 50 μm in panels a,b; 100 μm in panels c-j.